Nov. 7, 1990

Grant No: N00014-89-J-3073
Trimester Report, 7/1/90-11/7/90
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I. Work Summary

A. Purification of LPS binding factors in tolerant serum by affinity chromatography

Prior attempts in our laboratory to purify complexes of LPS formed by preincubation in tolerant serum utilized columns containing high affinity polyclonal IgG coupled to Sepharose beads. SDS-PAGE of eluted complexes revealed a tantalizing but elusive band of approximately 27KD. Repeated experiments occasionally gave inconclusive results. In order to remedy the situation and ameliorate our system we identified the following possible problems: 1. a short working half-life of the column due to elution of the complexes with 3M KSCN which rapidly denatured the bound anti-LPS IgG, 2. a long turn around time for experiments due to the need for multiple controls (normal and tolerant serum, with and without homologous and heterologous LPS) together with a relatively long concentration time for eluted samples using lyophilization, 3. some non-specific binding to the column despite relatively long wash times, and 4. the possibility that the columns did not contain sufficient anti-LPS IgG to capture adequate quantities of completed LPS.

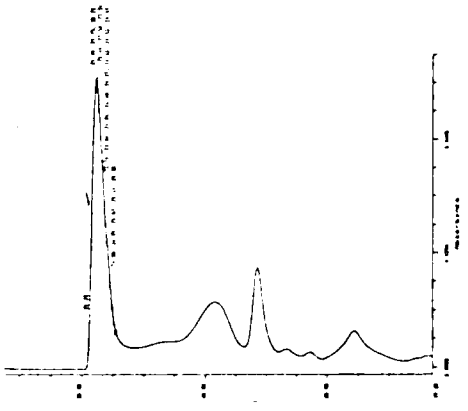
Accordingly, as a next generation of experiments, we coupled 17 milligrams of a high affinity murine monoclonal antibody specific for the O-polysaccharide side chain of E. coli 0111:B4 to sepharose 4B beads using cyanogen bromide, and prepared an affinity column containing 3.5 mls of gel. We are in the process of analyzing the results of the first experiments with the new system. To enhance column life, we are now eluting with glycine buffer at pH 2.8, rapidly followed by a phosphate buffer at pH 8.0. To decrease the time needed to concentrate eluted samples we obtained large volume rotors for our Speedvac rotary-vacuum concentrator. To decrease non-specific binding, if this is a problem with the new Mab column, we plan to add low concentrations of NaCl to our buffers and/or to stepwise elute with progressively higher concentrations of salt before the acid elution. We also plan to try non-ionic detergents. In each case we will monitor eluted LPS by radioactivity and by a double sandwich ELISA currently in routine use in the laboratory which is specific for the O-antigen of E. coli 0111:B4.

We are planning to convert this entire system over to HPLC. For the moment, however, we are in the midst of using the system for our other approach. We would also like to put off utilizing LPS in our tubing and UV monitor for as long as possible in the fear that we may have difficulty getting rid of it for pyrogen-free experiments.

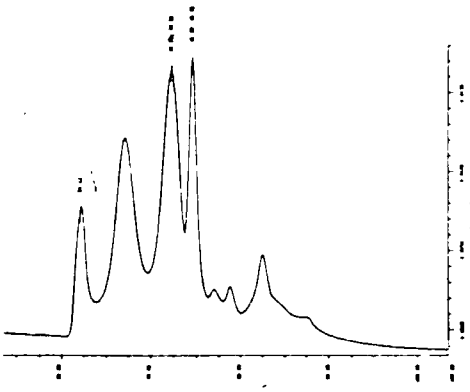
B. Purification of the LPS binding factor by ultracentifugation and chromatography using our radioimmunoassay

We have repeatedly confirmed that the activity of the factor (as measured by the precipitation of several different tritiated LPS) is found at density <1.21 g/ml in the lipoprotein density range. Accordingly our first purification step is ultracentrifugation in KBr to isolate the lipoproteins. In passing the purified lipoproteins from 5 mls of normal or tolerant serum over a 1 meter sepharose 6B-Cl we obtain 4 or 5 peaks at 280 nm optical density. There is consistently a second peak in the lipoproteins from the tolerant serum which is smaller or absent in the lipoproteins obtained from the normal serum control. We have been frustrated in the detection of activity in this peak, and the other peaks, because of the need to rapidly concentrate the samples and change buffers before testing. We have in a parallel set of experiments found that phosphate, Tris, and carbonate buffers unpredictably interfere with our assay system, presumably by complexing with the calcium in the calcium/dextran solution and variably trapping some tritiated LPS. We find that pyrogen-free saline adjusted to pH 7 works well. Previously, we have been hesitant to utilize microfiltration cells in order to avoid loss of the lipophilic material on the membranes and also to avoid LPS contamination. Accordingly, we have been concentrating the samples by absorption through dialysis tubing which works well but is time consuming. We have now started to concentrate using pyrogen-free tubes in large scale rotors in the Speedvac rotary-concentrator, which appears to work well. We are in the process of repeating our experiments with the modifications.

We have also converted to the HPLC system for the further purification of the lipoproteins. Several initial runs using a Superose 6 molecular sizing column (Pharmacia) revealed the chromatograms indicated below. We have much work to do changing chromatographic conditions in order to ameliorate resolution. Nonetheless, we were surprised and pleased to see that the chromatographic pattern of the lipoproteins from tolerant serum was indeed quite different from those from normal serum, even on a somewhat preliminary run. As on the low pressure sepharose 6B-Cl column, there was a peak eluting after the void volume in the tolerant lipoproteins which was small or absent in the normal lipoproteins.



Normal



Tolerant

C. Neutralization of LPS by tolerant serum as assessed by tumor necrosis factor production

In continuation of our studies involving LPS and the macrophage membrane, we have set up the assay as described in the original grant and measured the production of TNF by a murine macrophage cell line. LPS was incubated in each serum and then exposed to RAW 264.7 cells for four hours, at which time supernatants were harvested and assayed for TNF. Our results so far are shown below, and suggest to us that the LPS from some bacterial strains is neutralized more than others. We plan to continue and extend these studies because we feel that the interaction with LPS and the macrophage may be an integral part of the pathophysiology of endotoxic shock. We plan in particular to focus on the differences between K. pneumonia and P. aerugenosa in the assay. We also plan to study the effect of purified lipoproteins from normal and tolerant serum in the assay.

TNF production by RAW 264.7 cells induced with LPS incubated in normal and tolerant serum

		TNF (I.U.)	
LPS	Conc.	Normal	Tolerant
S. typhimurium	1 ng/ml	1100	16
		>6000	45
		425	150
K. pneumonia	1 ng/ml	900	14
		150	49
		2200	27
	10 ng/ml	2400	560
E. coli 0111:B4	1 ng/ml	530	190
	10 ng/ml	1300	420
E. coli 055	1 ng/ml	390	300
		105	42
P. aeruginosa	1 ng/ml	200	180
		28	90
		55	45
	10 ng/ml	430	170

II. New Knowledge

As noted above,

1. To circumvent certain technical difficulties with our affinity chromatography approach, it is desirable to make some changes in our procedure, one of which is the use of large quantities of a monoclonal antibody to capture the LPS complexes.

2. The chromatographic pattern of lipoproteins isolated from normal and tolerant serum is different, and there is a candidate peak present in those from tolerant serum and is not present in normal serum in our studies so far.

III. Technical Problems

None at this time. We have had minor problems over the last trimester, but have solved them as described above. We anticipate that we may encounter some difficulties as we launch into the mainstream of purifying the active lipoprotein component due to the marked hydrophobic nature of the substances which we are studying.

IV. Publications

We are hard at work on two manuscripts relevant to our work. Each is 90% completed and is referred to in the July 31 yearly summary.

V. Goals for the next trimester

a. Analysis of the LPS complexes from the monoclonal antibody column by SDS-PAGE, concentrating on the region where a 27KD band has been intermittently seen.

b. Evaluation of attempts to decrease non-specific binding in this system, if it is still a problem, using salt and detergents and varying elution conditions.

c. Continuation of the purification of lipoproteins on HPLC using our radioimmunoassay to detect activity. Amelioration of chromatography conditions. SDS-PAGE of relevant and active samples as appropriate. Use of Pharmacia HPLC columns based on hydrophobic interactions to see if we can capitalize on the properties of lipoproteins. ? use of ion exchange columns. Analysis of relevant samples for serum amyloid A (SAA) using ELISA or blotting with goat anti-SAA to see if this acute phase apolipoprotein is present in active samples.

d. Extension of studies on effect of tolerant serum on TNF production in our in vitro system. Study of isolated lipoproteins in this system, and study of active HPLC samples in the system.